

**γ -H2AX as a DNA damage marker to evaluate Suramin
sensitization effect on Cisplatin in pancreatic cancer xenograft**

By

PRACHI S. PATEL

DIVISION OF PHARMACEUTICS

College of Pharmacy

THE OHIO STATE UNIVERSITY

May 30, 2008

ADVISOR:

JESSIE L-S AU, PHARM.D., PH.D.

Abstract

Purpose: γ -H2AX, a DNA double strand break marker, was used to evaluate suramin sensitization effect in cisplatin-treated Capan-1-bearing mice xenografts.

Methods: Mice bearing pancreatic cancer Capan-1 subcutaneous xenografts were treated with control vehicle, cisplatin (7.5mg/kg), suramin (10mg/kg) combined with cisplatin, or suramin (100mg/kg) combined with cisplatin. Tumor samples were taken on 45th day after initiation of treatment. DNA damage after drug treatment was evaluated using immunohistochemical staining with γ -H2AX antibody.

Results: In terms of tumor growth, suramin at low dose enhanced tumor size shrinkage and delayed tumor regrowth compared to cisplatin alone, while high dose suramin antagonized cisplatin effect. Low dose suramin induced more DNA damage indicated by γ -H2AX positive staining; it is not significantly different from the cisplatin group though.

Conclusions: Present study identified the sensitization effect of low dose suramin on cisplatin-treated pancreatic cancer Capan-1 xenografts by inducing more tumor shrinkage and prolonged tumor regrowth delay. In addition, low dose suramin caused more DNA damage retaining indicated by higher level of γ -H2AX positive cells. This result serves as a preliminary study to use γ -H2AX as a DNA damage repair marker to evaluate suramin sensitization effect in DNA damaging agents

Introduction

Pancreatic cancer is a malignant tumor within the pancreatic gland. Pancreatic cancer is the 4th leading cause of cancer death for both men and women. Pancreatic cancer has been called a "silent" disease because in early stage pancreatic cancer usually does not cause symptoms. Cancer of the pancreas is curable only when it is found in its earliest stages, before metastasis appears. Patients have a poor prognosis in part because the disease is often locally advanced or metastatic at the time of diagnosis. The median survival time is less than 6 months. Pancreatic cancer is treated with surgery, radiation therapy, or chemotherapy. Researchers are also studying biological therapy to see whether it can be helpful in treating this disease. (4, 5, 6)

It has been known that Cisplatin is capable of causing DNA damage, and thus used in treatment of pancreatic cancer. Suramin has shown to act as a sensitizer to radiotherapy and chemotherapy at low dose (10-50 μ M). We carried out our experiment to find out whether suramin shows any sensitization effect on cisplatin treatment in pancreatic cancer. Cisplatin causes DNA damage by cross linkage usually at N-7 site of Guanine base. This results in DNA replication arrest and cell death if crosslink is not repaired. BRAC-2 is a gene that plays an important role in DNA break repair, but the cell line CaPan-1, which we have used is BRAC-2 deficient. Germline mutation in BRAC2 gene leads to the development of many other cancers such as Breast and Ovarian cancer along with Pancreatic Cancer. BRAC2 is human gene which is involved in repair of chromosome damage. (1, 3). Hence repair of DNA could not be assisted efficiently. In order to detect Double strand break in DNA, we used γ -H2AX as a double strand break marker. γ -H2AX by itself cannot repair the damage, but it acts as a recruiter, and

collects other repairing proteins to the localization where damage happens and completes the repair. After DNA damage is repaired, γ -H2AX experience degradation by phosphatases. Based on this knowledge we hypothesize that γ -H2AX can be used as a DNA damage marker to evaluate the cisplatin effect and further to evaluate the suramin sensitization effect on cisplatin. In current study, immunohistochemical staining was conducted using anti- γ -H2AX antibody to visualize the cells experiencing DNA damage, and the percentage of damaged cells in different groups was compared. (1,3)

Method

Cell Line

Capan-1 cell was purchased from ATCC. It is a hypo-triploid cell line derived from liver metastasis of a pancreatic cancer patient with a germline mutation in BRAC2. Tumor cells were maintained as monolayer culture at 37°C in a humidified atmosphere containing 5% CO₂ in IMDM (Iscoe's Modified Dulbecco's Medium) supplemented with 20% FBS, 4 mM L-glutamine, 90ug/ml gentamycin, and 90 ug/ml cefotaxime.

Chemicals and materials

γ -H2AX antibody was purchased from cell signaling technology. Kits of LSAB2 system-HRP and liquid DAB were purchased from DakoCytomation.

Animal and drug treatment protocols

Female athymic nu/nu mice (5-6 weeks old) had free access to sterilized rodent food and water *ad libitum*. Capan-1 cells (2 millioncells suspend in 100μl in a 1:1 solution of Matrigel and normal saline) were subcutaneously injected into bilateral sides of mice, and after 2 weeks, mice were randomized into 4 groups according to initial size. Cisplatin and suramin were dissolved in normal saline. Animals were treated with a single dose of

control vehicle, cisplatin (7.5 mg/kg), cisplatin + suramin (10 mg/kg) pretreatment, or cisplatin + suramin (100mg/kg) pretreatment at day 0. Cisplatin was given as single dose via i.v. injection, and suramin was given one day before the cisplatin treatment via i.p. injection. One month after the 1st cycle of treatment, the animals got the second cycle. The treatment schedule is the same as in the 1st cycle. Cisplatin (7.5 mg/kg) was given as single intravenous dose. Low dose suramin (10 mg/kg), or high dose suramin (100mg/kg) was given via i.p. injection a day before the cisplatin treatment. 10 days after the 2nd cycle of treatment, animals were anesthetized and tumor samples were harvested. Tumors were fixed, dehydrated, embedded in paraffin wax, cut to 5 μ m-thick sections, and stained for γ -H2AX.

Immunostaining

Immunostaining is a technique in which an antibody is used to link a cellular antigen specifically to a stain that can be more readily seen with a microscope. In our experiment γ -H2AX is an antigen in carcinoma and antibody is anti- γ -H2AX. Factors considered in designing our experiments include specimen source, antigen antibody affinity, antibody type, and detection enhancement methods. (2)

Fixatives Specimen (slide) preparation involves fixatives. They stabilize tissue thereby protecting them from the rigors of subsequent processing and staining techniques. We used Formalin as a fixative in our experiment. Fixation strengths and times were optimized so that antigens and cellular structures were retained and epitope masking could be reduced to minimal. (2)

Paraffin Sections Next we embed tissue in paraffin wax as it hardens tissue and provides excellent morphological detail and resolution. Because of their superior fidelity, clarity,

and preservational properties paraffin-embedded tissues have become the ultimate standard of immunohistochemistry in histology, and anytime where archiving of immunohistochemical information is required. (2)

Antigen Retrieval Antigen retrieval is one of the most important steps in immunostaining technique. Antigen Retrieval involves application of heat for varying lengths of time to paraffin-embed tissue sections in sodium citrate buffer. During this procedure the energy provided helps break some of the bonds formed during fixation, thus increasing the number of positive cells available, and intensity of reactions, although the exact mechanism is unclear. Therefore

antigen retrieval is done to maximize the availability of the antigen for interaction with a specific antibody. After a set time, the solution containing the slides is allowed to cool to room temperature slowly, and then slides are rinsed in PBS and used

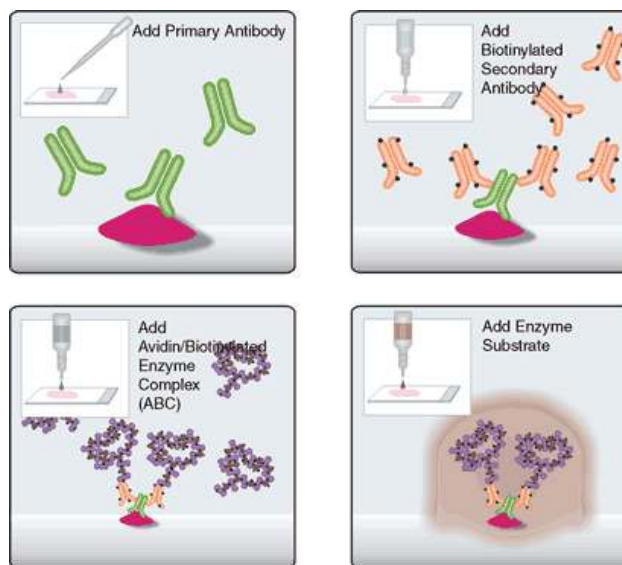


Figure 1 immunohistochemical staining protocol

for staining. (2)

Antibody Staining Further we used a procedure similar to standardized BrDU staining procedure. The proper working dilution for antibody was optimized. Dilution for our primary antibody anti- γ -H2AX was tested using 1:100 1:200, 1:400, and 1:800 dilution factors. The optimal antibody dilution is the one, which gives strongest specific antigen staining with the lowest non-specific background. In our experiment we got **1:400** as our

optimal antibody dilution. Biotinylated linker antibody solution (Yellow, containing anti-mouse immunoglobulins) and peroxidase-labelled streptoavidin solution (Pink) forms a conjugation along with the primary antibody. Staining is completed after incubation with substrate DAB-chromosome, which results in a brown colored precipitate at the antigen site. (2).

Enzyme-mediated Antibody Detection: When choosing a substrate for conversion by an enzyme, one should select a substrate, which yields a precipitating product. Enzyme involved in our experiment is HRP (Horseradish Peroxidase). HRP is specifically used for coloring thin slices of tissue biopsies for patients suspected to cancer. DAB (3, 3'-Diaminobenzidine) is the substrate used in our experiment which produces brown end product and is highly insoluble in alcohol and other organic solvents.

Cell Counting

We used Optimas microscopy to take pictures and to count cells. Percentage of γ -H2AX positive cells was determined as follows: Number of brown (antibody-bound) cells divided by sum of Brown and Blue cells, times 100. Blue colored cells are considered negative cells. Slides were counterstained with hematoxylin solution, thus contrast produced by blue colored cells benefited in identification of positive brown colored cells and in percentage counting of positive cells. (2)

Results

Suramin enhanced tumor shrinkage after combined with cisplatin treatment

After the 1st cycle of treatment, cisplatin alone suppressed tumor growth for about 7 days after the treatment, while addition of low dose suramin (10mg/kg) resulted in growth suppression for 11 days. At the 11th day, the average tumor sizes of these two groups

were 125% and 108% of the initial values, respectively. High dose suramin (100mg/kg) didn't cause shrinkage of the tumor size or delay tumor growth compared to cisplatin alone treatment. The treatment of the 2nd cycle delayed the tumor progression for less than 1 week, and animals in all 3 groups started regrowing 1 week after the treatment. Addition of low dose suramin didn't further prolong the tumor suppression duration. High dose suramin still showed obvious antagonistic effect.

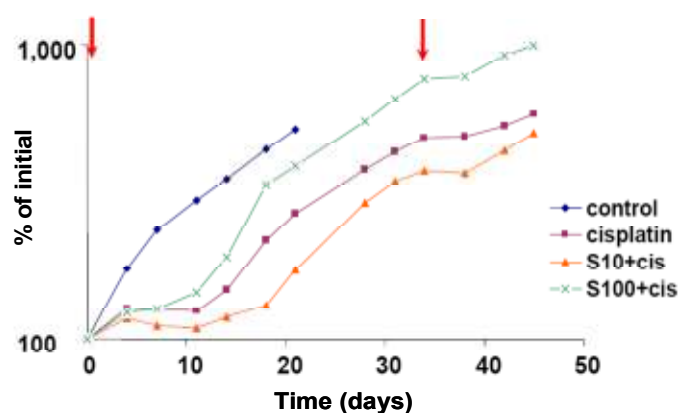


Figure 2 Tumor growth curve after treatment

Suramin might have delayed DNA damage repair after treatment

The presentative pictures of each group were shown in Figure 3 and red circles indicated the γ -H2AX positive cells, which suggested that the cells were damaged after drug treatment and the damage was not repaired at the time of anesthetization.

The percentage of γ -H2AX positive cells was summarized in Table 1 and plotted in Figure 4 as bar graphs. The results showed that the groups of cisplatin, Suramin10+Cisplatin and Suramin100+Cisplatin had significant higher number of positive stained cells compared to Control. Suramin10+cisplatin had higher γ -H2AX positive cells than cisplatin alone and suramin100+cisplatin groups, but the difference is not statistically significant (p values are shown in Table 2).

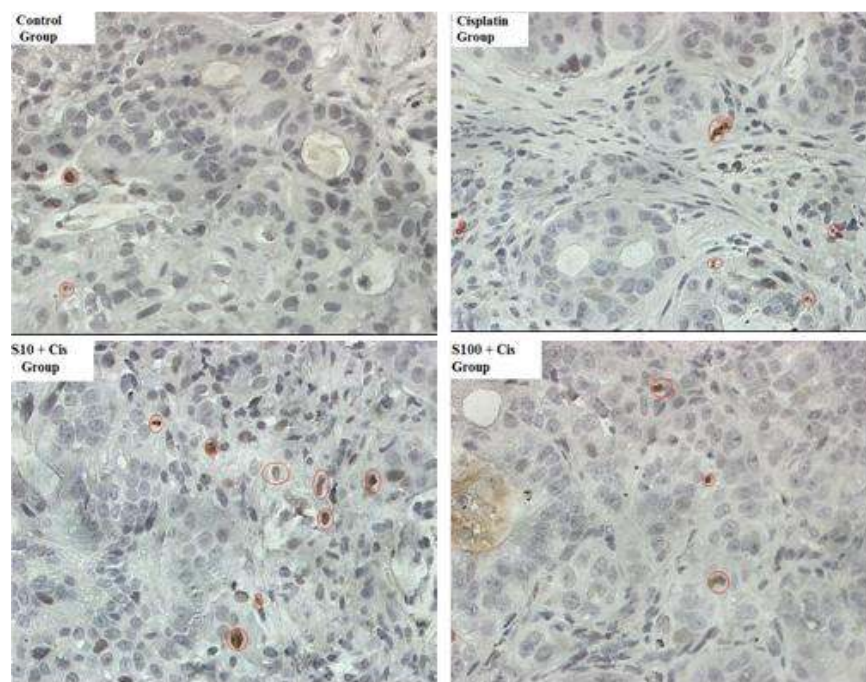


Figure 3. Representative γ -H2AX staining pictures in each groups

Table 1. Percentage of γ -H2AX positive stained cells in each group

	Control	Cisplatin	Suramin10 + Cisplatin	Suramin100 + Cisplatin
Positive Stained Cells (%)	1.10	3.21	4.65	3.45
Standard Deviation (\pm)	0.49	1.89	2.72	1.71

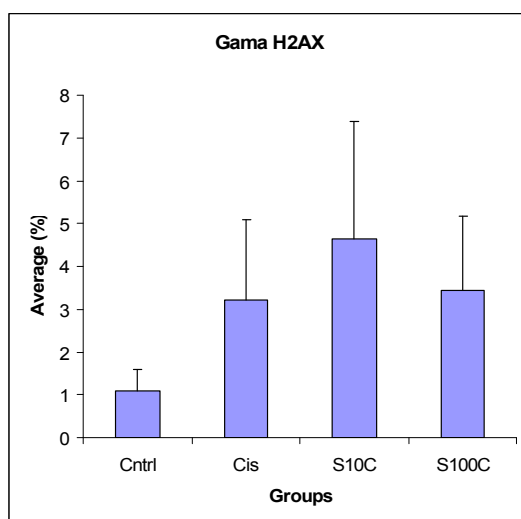


Figure 4. Graphical representation of γ -H2AX positive cells.

Cntrl: control

Cis: cisplatin

S10C: suramin 10 + cisplatin

S100C: suramin 100 + cisplatin

Table 2. summary of p-values

	Cis Vs. Ctl	S10C Vs. Ctl	S100 Vs. Ctl	S10C Vs. Cis	S100C Vs. Cis
P- value	0.010054	0.009182	0.012898	0.207561	0.423794

Conclusion

Present study identified the sensitization effect of low dose suramin on cisplatin-treated pancreatic cancer Capan-1 xenografts by inducing more tumor shrinkage and prolonged tumor regrowth delay. In addition, low dose suramin caused more DNA damage retaining indicated by higher level of γ -H2AX positive cells. This result serves as a preliminary study to use γ -H2AX as a DNA damage repair marker to evaluate suramin sensitization effect in DNA damaging agents.

Discussion

Our study tested the feasibility of using γ -H2AX, the DNA double strand break marker, as an Pharmacodynamic endpoint to evaluate suramin sensitization effect. Low dose suramin did cause more unrepaired damage compared to cisplatin alone, but the difference is not statistically significant. The possible reasons might include the following:

1) Limitation of sample size. As shown in figure 4, we got big variation for data set, which is due to heterogeneity of animal tumor samples. To decrease standard deviation, we could increase the number of observations by taking and counting more randomly selected pictures for each sample.

2) *in vivo* experiment setting. γ -H2AX was reported to peak 24 hours after radiation treatment (7), and start to be degraded after that. So we can think that activation of γ -H2AX is a fairly early effect after DNA damage, and once the damage is repaired

sufficiently, the level of γ -H2AX will drop. In current experiment, the tumor samples were got after 2 cycles of treatments. As shown in Figure 1, tumors had regrown to fairly large sizes after the 1st cycle treatment, and the 2nd cycle of treatment didn't cause as much tumor shrinkage as the 1st cycle did, possibly due to the acquired resistance, It is highly possible that the damage caused by 1st cycle treatment had been repaired, while the 2nd cycle didn't cause much damage due to resistance. That could explain why the percentage of damaged cells was less than 5% in all groups, and the difference between groups was too small to be significant. Further study with earlier sampling time is expected to give a more clear result.

The current study provides preliminary data to test the hypothesis that γ -H2AX can be used as a DNA damage marker to evaluate suramin sensitization effect in cisplatin treated pancreatic capan-1 xenografts. Further study is needed to support the hypothesis.

Awareness

- November is Pancreatic Cancer Awareness Month
- Purple is the traditional color chosen to represent pancreatic cancer awareness.
- Research spending per pancreatic cancer patient is \$1145, the second lowest of any leading cancer.
- The Pancreatic Cancer Action Network (PanCAN) was created as an advocacy group for pancreatic cancer.

References

1. Goggins M, Schutte M, Lu J, Moskaluk CA, Weinstein CL, Petersen GM, Yeo CJ, Jackson CE, Lynch HT, Hruban RH, Kern SE. Germline BRCA2 gene mutations in

patients with apparently sporadic pancreatic carcinomas. Cancer Research. 1996 Dec 1; 56(23):5360-4.

2. Immunostaining Techniques, Immunohistochemistry / Immunocytochemistry. Web Source : <http://www.millipore.com/immunodetection/id3/immunostainingtechniques>

3. Molecular Cytogenetics of Common Epithelial Cancers, Pancreatic Carcinoma cell lines, Capan-1 Web source :

<http://www.path.cam.ac.uk/~pawefish/PancCellLineDescriptions/capan-1.html>

4. U.S. National Institute of Health, National Cancer Institute. Web Source : www.cancer.gov

5. Cancer Center. Pancreatic Cancer, the Silent Disease. Web Source: <http://www.medicinenet.com/script/main/art.asp?articlekey=84931>

6. Pancreatic cancer-Symptoms, treatment, Prevention. Web Source: [**http://www.healthscout.com/ency/1/597/main.html**](http://www.healthscout.com/ency/1/597/main.html)

7. **Judit P. Banáth, Susan H. MacPhail and Peggy L. Olive**, Radiation Sensitivity, H2AX Phosphorylation, and Kinetics of Repair of DNA Strand Breaks in Irradiated Cervical Cancer Cell Lines. Cancer Research 64, 7144-7149, October 1, 2004